

## Original Article

# The effect of the carotenoid bixin and annatto seeds on hematological markers and nephrotoxicity in rats subjected to chronic treatment with cisplatin



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## ABSTRACT

This study assessed the protective effect of the carotenoid bixin and annatto seeds against possible nephrotoxicity induced with a single peritoneal administration of pharmacological cisplatin in male Wistar rats. After 48 h, the blood cell differential count showed a significant reduction in neutrophil counts in rats that received a diet rich in bixin when compared to the group that received only cisplatin. The use of cisplatin led to an increase in kidney weight. The carotenoid bixin attenuated renal injury, characterized by increased polymorphonuclear infiltration. No protective effect was observed with respect to Annatto. These results demonstrate the role of toxic cisplatin and suggest that bixin affords a protective effect against cisplatin-induced nephrotoxicity in adult Wistar rats.

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## Introduction

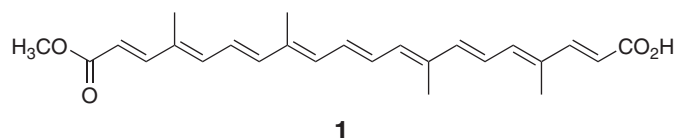
Cisplatin [*cis*-diamminedichloroplatinum(II) – CDDP] is a chemotherapeutic agent used alone or in combination with other antineoplastic agents in the treatment of lung, brain, throat, esophagus, stomach, colon, bladder, testicular, ovarian and uterine and other cancers. However, cisplatin nephrotoxicity is the principle dose-limiting factor for the use of cisplatin (Santos et al., 2012). It has been suggested that the generation of reactive oxygen species and lipid peroxidation is responsible for the cisplatin-induced renal tubular impairment (Chirino and Pedraza-Chaverri, 2009).

The physiological functions of patients undergoing chemotherapy should be monitored using periodic laboratory tests to analyze parameters of global renal function, such as creatinine and urea, and obtain the blood cell count to determine general immune status (Sodré et al., 2007).

Carotenoids are compounds that have antioxidant properties capable of sequestering free radicals. They are found in various foods and may have a protective effect against oxidative damage

caused, for example, by chemotherapy (Antunes and Bianchi, 2004; Rios et al., 2009; Bautista et al., 2004).

Annatto is a natural color extracted from the outer coating of the seeds of the Annatto tree (*Bixa orellana* L., Bixaceae), which is native to the Amazon region in South America (Mercadante, 2001; Gomes, 2007). Its color varies from yellow to red, and the most abundant carotenoid is *trans*-bixin (1), which accounts for around 80% of the total pigment content of the seeds (Mercadante, 2001; Giuliano et al., 2003; Bautista et al., 2004). In addition to its coloring action, annatto also has functional properties that form the basis of a variety of roles and actions in living organisms due to the presence of antioxidant compounds (Krinsky, 1994). Studies have shown that bixin has beneficial effects, including the reduction of oxidative stress and its ability to act as a chemo-preventative agent and to reduce the nephrotoxic effects of antitumor agents (Bertram and Bortkewicz, 1995; Agner et al., 2005; Rios et al., 2009).



In an attempt to minimize the side effects caused by cisplatin, this study evaluates the antioxidant activity of the carotenoid bixin

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and annatto seeds on cisplatin-induced nephrotoxicity in adult male Wistar rats by examining biochemical and histological aspects to assess renal function and morphology and performing complete blood counts.

## Materials and methods

### Plant material

This study examined annatto (*Bixa orellana* L., Bixaceae) from the Eldorado access, ICN (189644) 10.VII.2009, collected and identified by J.M. Wiest.

### Assays

#### Animals

The biological assay was performed in the Vivarium of the Food Science Department of the Institute of Food Science and Technology of the Federal University of Rio Grande de Sul in Brazil. The animals were housed in ventilated rooms at a temperature of  $24 \pm 2^\circ\text{C}$  with a 12–12 h light–dark cycle and  $60 \pm 5\%$  humidity and given *ad libitum* access to food and water.

The animals were divided at random into four groups: CG (control group), CPG (cisplatin group), CPBG (cisplatin/bixin group), and CPAG (cisplatin/annatto group). The biological trial lasted 28 days. The CG group received commercial rodent feed, the CPG group received commercial feed plus cisplatin ( $5\text{ mg kg}^{-1}$  body weight), the CPBG group received commercial feed with bixin ( $0.065\text{ mg kg}^{-1}$  body weight) and cisplatin ( $5\text{ mg kg}^{-1}$  body weight), and the CPAG group received commercial feed with cisplatin ( $5\text{ mg kg}^{-1}$  body weight) and annatto ( $0.500\text{ mg kg}^{-1}$  body weight). The doses of bixin and annatto used in pretreatment were based on data found in the literature on nephroprotective effects (FAO/WHO, 2007; Antunes and Bianchi, 2004). A single dose of cisplatin ( $5\text{ mg kg}^{-1}$ ) was administered 48 h before the end of the experiment to induce oxidative stress.

#### Obtaining annatto and bixin crystals

Bixin crystals were obtained using a methodology developed by Rios and Mercadante (2004). The seeds were first washed in hexane and methanol to remove hydrophilic and hydrophobic impurities and the bixin was then extracted using ethyl acetate. The extract obtained through this process was dried in a rotary evaporator ( $T < 30^\circ\text{C}$ ) and rediluted in dichloromethane. For the crystallization process, the extract was heated on a hot plate ( $T < 50^\circ\text{C}$ ). After being chilled, absolute ethanol was added and the extract was then cooled in an ice bath and placed in a freezer at  $14 \pm 2^\circ\text{C}$  for 24 h. The bixin crystals were filtered and washed with chilled absolute ethanol, dried in a vacuum oven for 24 h, and then stored at  $-18^\circ\text{C}$  until use.

#### Purification of bixin

The purity of bixin was determined using a method described by Tocchini and Mercadante (2001), in which the bixin is extracted from annatto seeds previously washed with petroleum ether and a mixture of methanol and dichloromethane (1:1). After concentration, the extract was successively purified by silica gel thin layer chromatography using both ethyl acetate and petroleum ether (3:2) as mobile phases. In the first chromatography, silica gel layers (Merck) were prepared in the laboratory and the two separated bands and everything remaining at the origin were scraped off and eluted with the methanol/dichloromethane mixture (1:1). After concentration, the band was applied again to ready silica plates and split into two bands. The major one at  $R_f$  0.61 was scraped off and

eluted with the methanol/dichloromethane mixture. The pattern obtained shows 99% purity, checked by High Performance Liquid Chromatography (HPLC) (Agilent 1100 series).

#### Cisplatin

Cisplatin [cis-diamminedichloroplatinum (II), CDDP; CAS N<sup>o</sup>. 15663-27-1] was kindly donated in its commercial form by Quiral Química do Brasil S.A. (Platinil<sup>®</sup>).

#### Blood collection and doses of urea and creatinine

The animals were sedated for blood collection using benzodiazepine ( $0.25\text{ mg } 100\text{ g}^{-1}$  body weight) and sodium pentobarbital ( $4.6\text{ mg } 100\text{ g}^{-1}$  body weight). A median incision was made in the ventral part of the abdomen and blood was collected from the ascending aorta. The blood was centrifuged for 10 min at  $5081.31 \times g$  force to separate serum for urea and creatinine analyses.

Urea and creatinine levels were determined using commercial kits (Bio Liquid). Urea was quantified using a spectrophotometer (Micronal – B342II digital model) adopting the UV kinetic method, while creatinine was quantified using the colorimetric kinetic method. The results were expressed in mg/dl.

#### Blood cell differential count

The blood collected from the aorta was placed into flasks containing EDTA solution. The animals were subsequently sacrificed. The blood smears were stained (Instant Prov – New Prov) and manually analyzed to determine the blood cell differential count using an optical microscope. The blood cell differential count was expressed as a percentage (%).

#### Determination of kidney weight and preparation for histological analysis

The kidneys were washed free of blood (by perfusion) with a saline solution. A fixing solution was then added and the kidneys were weighed using a Micronal B 600 balance and fixed using Bouin's solution for 24 h at room temperature.

The material was prepared for histological analysis using routine techniques (Prophet et al., 1994). After fixing for 24 h, the sample tissues were cryoprotected using sucrose solutions at increasing concentrations (from 15% to 30%) and then frozen in nitrogen. Next, using a cryostat (Leitz, Digital 1702, Germany) at  $-20^\circ\text{C}$ , each piece was cut at  $15\text{ }\mu\text{m}$  thickness intervals and the slice were placed on glass slides and stained using hematoxylin and eosin (HE) to assess tissue morphology. Histological parameters were obtained using an Olympus<sup>®</sup> Bx50 optical microscope (Olympus, Tokyo, Japan).

#### Ethical aspects

This study was approved by the Animal Research Ethics Committee of the Federal University of Rio Grande do Sul (reference number 17809), being considered ethically and methodologically adequate according to Resolution 196/1996 and complementary items of the Brazilian National Health Council.

#### Statistical analysis

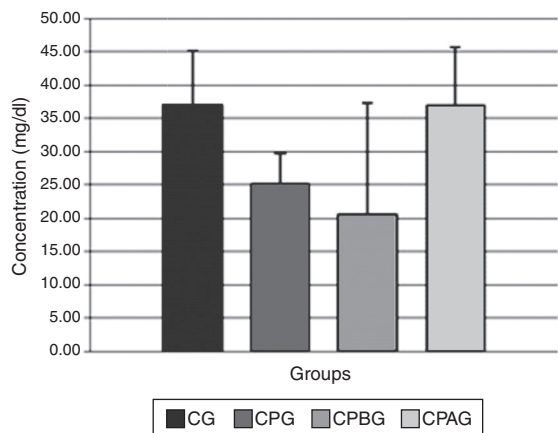
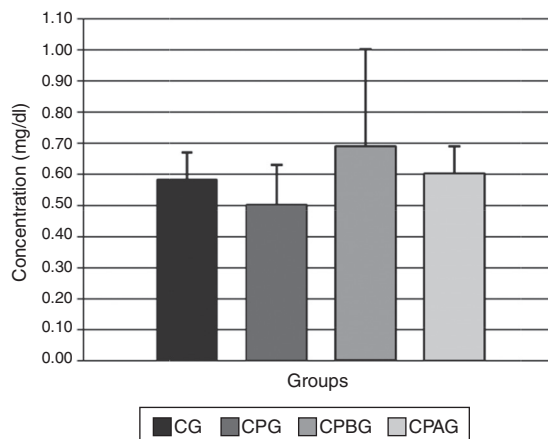
Data was submitted to an analysis of variance (ANOVA) to detect significant differences between study groups. Tukey's test was applied to identify any difference between means using a significance level of  $p \leq 0.05$ .

**Table 1**

Means and standard deviations of blood cell differential counts in Wistar rats subjected to different treatments.

	CG (n=6)	CPG (n=6)	CPBG (n=6)	CPAG (n=6)
Neutrophils (%)	16.5 ± 4.9	24.6 ± 9.2 <sup>a</sup>	13.5 ± 3.5 <sup>a</sup>	18.33 ± 3.7
Lymphocytes (%)	73.3 ± 3.9	67.4 ± 9.0	75.5 ± 5.7	72.50 ± 3.2
Monocytes (%)	8.0 ± 2.7	7.8 ± 1.9	10 ± 3.6	9.0 ± 4.1
Basophils (%)	0.2 ± 0.4	0.2 ± 0.4	0.3 ± 0.8	0.16 ± 0.4
Eosinophils (%)	1.3 ± 1.0	0.4 ± 0.9	0.3 ± 0.8	0 ± 0

CG, control group; CPG, cisplatin group; CPBG, cisplatin/bixin group; CPAG, cisplatin/annatto group.

<sup>a</sup> CPG statistically different from CPBG ( $p < 0.05$ ).**Fig. 1.** Mean and standard deviation of blood urea dosing (mg/dl) in Wistar rats 48 h after administering cisplatin. Control group (CG), cisplatin group (CPG), cisplatin/bixin group (CPBG) and cisplatin/annatto group (CPAG),  $p > 0.05$ .**Fig. 2.** Mean and standard deviation of blood creatinine dosing (mg/dl) in Wistar rats 48 h after administering cisplatin. Control group (CG), cisplatin group (CPG), cisplatin/bixin group (CPBG), and cisplatin/annatto group (CPAG),  $p > 0.05$ .

## Results and discussion

With respect to hematological analysis, the blood cell differential count showed a significant reduction in neutrophil counts ( $p < 0.05$ ) in the group that received a diet rich in bixin ( $13.5 \pm 3.5\%$ ) when compared to the group that received only cisplatin ( $24.6 \pm 9.2\%$ ). No significant difference was found between the groups ( $p > 0.05$ ) with respect to other blood cells: lymphocytes, monocytes, basophils and eosinophils (Table 1). The immunological response can be divided into adaptive (innate immune) and non-adaptive components, enhancing the phagocytic capacity of cells, such as monocytes, macrophages and neutrophils, which act in the destruction of antigens and constitute the first line of human defense mechanisms (Pathak and Palan, 2005).

In given situations, particularly in the case of acute inflammation, high levels of neutrophils occur. This was observed in the present study; however, when the group that received cisplatin was compared with the group that received cisplatin/bixin, it is evident that bixin apparently minimized the inflammatory effects of cisplatin, given that it significantly ( $p < 0.05$ ) reduced the levels of neutrophils, indicating a reduction in antitumor agent-induced inflammation.

No statistically significant difference was found between the groups, with respect to dose in relation to plasma urea levels, although a tendency ( $p = 0.07$ ) toward decreased urea levels was observed in the group receiving dietary bixin/cisplatin in comparison to the CG and group receiving annatto/cisplatin, as shown in Fig. 1.

It is important to evaluate plasma creatinine and urea levels together, despite the fact that these two parameters do not always show concomitant high levels. In cases of prerenal attack, situations such as protein-rich diet, high protein breakdown rates, and dehydration, high levels of urea may be found together with normal creatinine levels. However, these two dosages change together

in situations of postrenal attack, where renal obstructions, malignancy, renal excretion disorders and a decrease in the glomerular filtration rate occur (Burtis et al., 2006).

Urea results from the catabolism of the amino acids, and is eliminated from the body, predominantly by the kidneys (Burtis et al., 2006). Urea is synthesized exclusively by hepatic enzymes and it is a very unstable marker, since it is influenced by the protein content of diets, and thus it is not as accurate as creatinine for evaluating renal function. However, it serves as a complementary indicator in the diagnosis of renal injury (Sodré et al., 2007; Burtis et al., 2006).

No significant difference between the groups was found ( $p > 0.05$ ) with regard to serum creatinine dosage (Fig. 2). Serum creatinine dosage is the most commonly used indicator for monitoring the evolution of renal failure, since it is the least variable nitrogenous compound in the blood. Since it is a residue of creatinine, free creatinine is not reused by the body's metabolism, but rather excreted through glomerular filtration (Lima et al., 2003).

The macroscopic examination of the kidneys of rats from all groups showed that they had a normal appearance, with a pink-red color and firm consistency. The results of kidney weight in relation to body weight revealed possible swelling of this organ, which could be related to local inflammatory processes (Table 2).

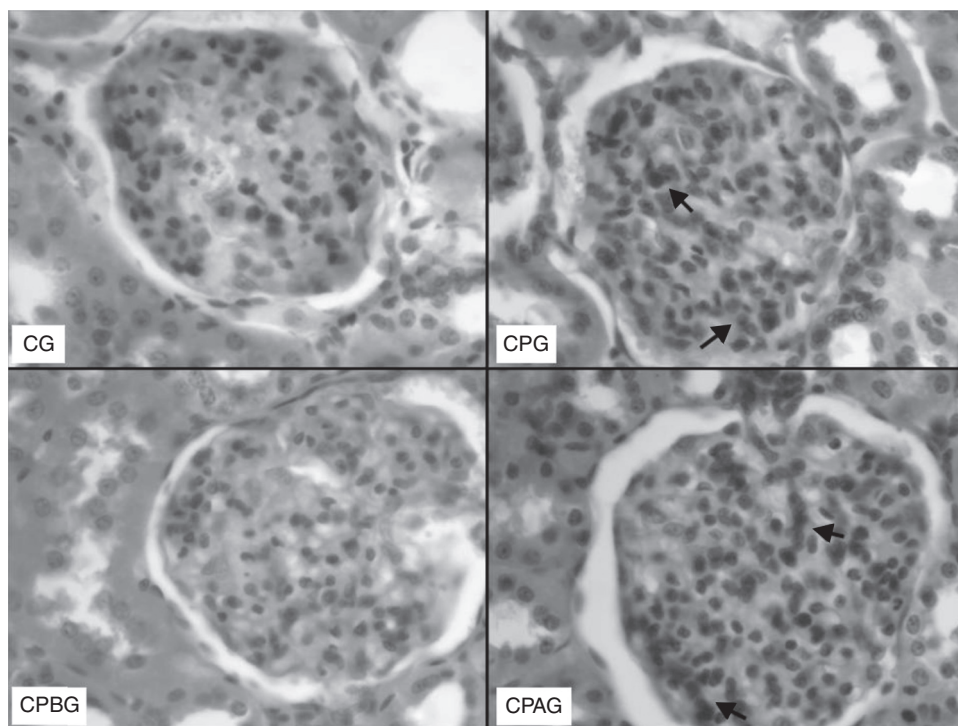
In the group that received cisplatin, the substance effectively inhibited an increase in kidney weight – which generally follows the same pattern as that of body weight – which was statistically lower in the animals in this group. There was no statistically significant difference between the bixin group and the CPG (Table 2). The results observed in this study corroborate the findings of other studies that observed an increase in the kidney–body weight ratio in rats treated with cisplatin (Saad et al., 2004). It has been suggested that an increase in kidney weight in rats is associated with nephrotoxicity related to tubular epithelial necrosis caused by cisplatin (Cadernas and Barja, 1999). A relative increase in kidney weight and histological changes, together with an increase in the

**Table 2**

Mean and standard deviation of the “final body weight/kidney weight” ratio in Wistar rats subjected to different treatments.

	<i>n</i>	Initial weight (g)	Final weight (g)	Renal weight (g)
CG	6	250.31 ± 13.80	375.71 ± 24.81	1.36 ± 0.10
CPG	6	244.81 ± 16.90	369.42 ± 21.48	1.41 ± 0.21
CPBG	6	249.11 ± 16.70	353.61 ± 10.30	1.38 ± 0.10
CPAG	6	253.01 ± 28.80	347.11 ± 24.89	1.23 ± 0.10 <sup>a</sup>

CG, control group; CPG, cisplatin group; CPBG, cisplatin/bixin group; CPAG, cisplatin/annatto group.

<sup>a</sup> CPG statistically different from CPBG ( $p < 0.05$ ).**Fig. 3.** Photomicrographs of hematoxylin and eosin stained kidney sections of rats in the control group (CG), cisplatin group (CPG), cisplatin/bixin group (CPBG), and cisplatin/annatto group (CPAG), showing polymorphonuclear infiltrates (arrow). Magnification 400×.

number of inflammatory cells, were noted in animals that received only cisplatin, suggesting a similar association in this group.

The histological analysis of the kidney sections showed that characteristic structures were forming in this organ. The presence of glomeruli was noted in the cortical region, surrounded by twisted proximal and distal tubules (Fig. 3).

The renal tissue of the animals that received cisplatin showed polymorphonuclear (PMN) infiltrates in the glomerular region, which is a characteristic of inflammatory changes. However, PMN infiltrates were not present in the kidneys of the animals that received pretreatment with bixin (Fig. 3).

PMN, attracted during the reperfusion process, are considered important mediators during the beginning phase parenchymal injury in cases of acute ischemic renal failure. On the other hand, the rate of infiltration of mononuclear leukocytes can vary depending on renal damage (Ysebaert et al., 2004).

According to Faraco (1996), the number of PMN within the glomerular capillaries is an indicator of the level of inflammatory activity in glomerulopathies, including acute glomerulonephritis (Oliveira, 2005).

Acute glomerulonephritis, in addition to the presence of macrophages and PMN, is characterized by increased glomerular volume and a proliferation of the mesangial cells (Faraco, 1996; Oliveira, 2005). Glomerular changes were observed in the kidneys of the CPG, characterized by PMN infiltrates, which could indicate acute glomerulonephritis. However, PMN infiltrates were not

present in the kidneys of animals who received pretreatment with bixin, which suggests that this substance may afford protection against inflammation typically caused by this antitumor agent.

These results corroborate the findings of other studies concerning damage caused by cisplatin. A study conducted by Behling et al. (2006) evaluating the effect of multiple doses of quercetin to inhibit renal damage caused by cisplatin showed that the administration of this flavonoid prevents and significantly reduces renal damage, and that treatment with cisplatin without quercetin increases plasma creatinine levels, lipid peroxidation and renal damage, as shown by the histological analysis of this organ.

The histological analysis of kidney sections from the group that received cisplatin/annatto showed that this group did not receive the same level of protection as that afforded in the bixin group, despite a reduction in kidney weight. This difference could be explained by the dosage adopted by the present study, which may have been excessive. In a study evaluating the protective effect of annatto and bixin against the genotoxicity of cisplatin in PC12 cells using the micronucleus test, Santos et al. (2012) verified that 2.0, 3.0 and 4.0 µg/ml concentrations of annatto induced genotoxic damage to the PC12 cells. However, pretreatment with 0.2, 0.5 and 1.0 µg/ml concentrations of annatto resulted in a protection rate of between 66.4% and 76.7% against chromosomal damage induced by cisplatin. Some carotenoids, when used in very high doses, fail to present the desired effect and can act as pro-oxidants (Behling et al., 2006; Rios et al., 2009).



With respect to the proximal and distal tubules, no differences were observed between the groups, showing that neither cisplatin nor pretreatment with bixin and annatto had any effect. These results are contrary to the findings of Behling et al. (2006) who, using the same dose of cisplatin adopted in the present study (5 mg/kg), noted morphological changes such as tumor-tubular necrosis. However, the length of exposure to the drug in the study carried out by Behling was longer than that of the present study (5 and 20 days, as compared to just 2 days, respectively).

## Conclusions

The findings of this study suggest that exposure time was sufficient to demonstrate that cisplatin caused acute injury and that bixin probably helped to minimize inflammation, as shown by the low levels of neutrophils.

However, pretreatment with bixin attenuates kidney damage, preventing an increase in PMNs, and is a possible protector against cisplatin-induced acute inflammatory processes.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

**Confidentiality of data.** The authors declare that they have followed the protocols of their work center on the publication of patient data.

**Right to privacy and informed consent.** The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

## Authors' contribution

LFS, CHP and NSM (PhD student) contributed to organizing the laboratory work, data analysis and drafted this paper. SB, PCPS and MA contributed to the biological studies. EVJ and AOR were responsible for study design, supervised the laboratory work and made a critical reading of the manuscript. We confirm that the manuscript has been read and approved by all named authors.

## Conflicts of interest

The authors declare no conflicts of interest.

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